The novel thermogenic β -adrenergic agonist Ro 16-8714 increases the interscapular brown-fat β -receptor–adenylate cyclase and the uncoupling-protein mRNA level in obese (*fa/fa*) Zucker rats

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In obese (*fa/fa*) rats both the total (–)-isoprenaline- and NaF-stimulated adenylylase activities of interscapular brown-adipose-tissue (IBAT) plasma membranes were decreased as compared with lean rats by 40 and 38 % respectively. An acute treatment with Ro 16-8714 increased (–)-isoprenaline- and NaF-stimulated adenylylase activities by 2.5- and 2.0-fold respectively, and β -adrenoceptor number 2.8-fold in obese rat IBAT, but it had no effect on these parameters in lean rats. It increased mRNA for mitochondrial uncoupling protein in both lean and obese rats to the same extent.

INTRODUCTION

Brown adipose tissue (BAT), the main effector of cold- and diet-induced thermogenesis in rodents [1,2], is under the control of the sympathetic nervous system [3], which acts through β -adrenergic receptors [4].

Several abnormalities of BAT have been reported to be associated with obesity in (*fa/fa*) Zucker rats. Nor-adrenaline content and turnover [5,6] were decreased, whereas β -adrenergic receptor density was found to be unaffected in young [7] and decreased in adult [8,9] rats. Mitochondrial uncoupling protein (UCP) [10] and GDP-binding sites [11], which are considered an index of thermogenic activity, were also decreased.

The novel thermogenic β -agonist Ro 16-8714, when administered *in vivo*, inhibits body-weight gain and increases BAT GDP-binding sites in obese (*fa/fa*) Zucker rats [12]. Recently, the effects on interscapular BAT (IBAT) of an acute treatment with Ro 16-8714 were studied in obese *fa/fa* rats [13]. The results showed a sharp decrease in tissue wet weight and an increase in total plasma-membrane proteins. The current study was undertaken to investigate whether this increase in the amount of plasma-membrane protein was correlated with an increase in the efficiency of the plasma-membrane β -adrenergic effector system, i.e. the β -receptor–adenylate cyclase couple.

Thus the effects of an acute treatment with Ro 16-8714 on binding of the β -adrenergic ligand [¹²⁵I]cyanopindolol and on basal and stimulated adenylylase activities of IBAT plasma membranes were measured in lean and obese (*fa/fa*) rats. A more distal effect of Ro 16-8714, i.e. that on UCP mRNA level, was also studied in the same animals.

MATERIALS AND METHODS

All organic and inorganic chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany), Sigma (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland). [α -³²P]ATP (410 Ci/mmol), [α -³²P]-dCTP (6000 Ci/mmol) and [¹²⁵I]cyanopindolol (2000 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). Ro 16-8714 was a gift from Hoffmann–La Roche (Basle, Switzerland). Membrane filters (Nytran Ny 13) were obtained from Schleicher and Schuell (Feldbach, Switzerland).

Female lean (*Fa/?*) and obese (*fa/fa*) Zucker rats (9 weeks old) were kept at room temperature (about 21 °C) with 12 h of illumination per day and fed *ad libitum* with Provimi Lacta chow (Cossonay, Switzerland). The animals were dosed orally with either Ro 16-8714 (2 μ mol = 0.87 mg/kg) in 5 % (w/v) gum arabic (10 ml/kg) or with the vehicle alone at 30 h and 6 h before they were killed. The IBAT plasma membranes were prepared from pools of IBAT from six rats as previously described [14], except that for obese-rat IBAT the fat was re-homogenized three times in 10 ml of homogenizing medium at room temperature to improve the recovery of adsorbed subcellular membranes. The 5'-nucleotidase activity was measured as described previously [15]. Protein concentrations were determined by the method of Lowry *et al.* [16].

In binding studies, the plasma membranes were incubated for 30 min at 37 °C in 20 mM-phosphate buffer, pH 7.4 (total volume 0.5 ml) containing various concentrations of [¹²⁵I]cyanopindolol (10, 25, 40, 50, 75, 100, 150, 200, 250 pM). The binding of the radioligand to the membranes was determined by the poly(ethylene glycol)

Abbreviations used: BAT, brown adipose tissue; IBAT, interscapular BAT; UCP, mitochondrial uncoupling protein.

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precipitation technique [17]. Specific binding was defined as the difference between total binding obtained in the absence of competing ligand and the non-specific binding value obtained in the presence of 10 μM (–)-propranolol. The adenylate cyclase activity was measured by the technique described by Salomon *et al.* [18] as modified by Cooper *et al.* [19], alone or in the presence of 10 mM-NaF or of various concentrations of (–)-isoprenaline (0.1, 0.5, 2.5, 10, 50 μM). The stimulated activity values were obtained by subtracting the basal value measured in the presence of 10 μM -GTP alone from that measured in the presence of GTP+NaF or of GTP+(–)-isoprenaline. In both binding and adenylate cyclase assays the amount of protein used was within the ranges (20–50 and 10–20 μg per assay respectively) in which the activities were found to increase linearly with the protein concentration. The quantitative parameters K_D and B_{max} of specific [^{125}I]cyanopindolol binding were determined by Scatchard-plot analysis [20] by using the LIGAND program [21]. K_{act} and V_{max} for adenylate cyclase activity were calculated by non-linear-regression analysis.

Total IBAT RNA was isolated by the phenol procedure, electrophoresed in an agarose gel containing formaldehyde, fixed to Nytran Ny 13 membranes by Northern blotting and hybridized with ^{32}P -labelled cloned pUCP 36 insert as described previously [22]. To check the specificity of changes in amount of UCP mRNA, the Northern blots were also hybridized with a ^{32}P -labelled pST 41 total mouse mitochondrial DNA probe (gift from Dr. H. Blanc, Université Paris-Sud, Orsay, France). Densitometric analysis of autoradiograms was performed with a high-resolution laser-beam densitometer.

RESULTS

In 9-week-old lean rats, the IBAT wet weight was 300 ± 17 mg ($n = 5$). It was 2.7-fold heavier ($P < 0.001$) in obese rats. Ro 16-8714 acute treatment decreased IBAT wet weight by 24% ($P < 0.001$) and by 40% ($P < 0.001$) in lean and obese rats respectively. In lean and obese rats, the amount of plasma-membrane protein obtained from one IBAT was 345 ± 44 and 287 ± 30 μg ($n = 5$; no significant difference). Ro 16-8714 treatment, although it reduced IBAT wet weight, did not change the amount of plasma membranes obtained from one IBAT in lean rats, but increased it 1.6-fold ($P < 0.005$) in obese

rats. In all four groups the specific activity of the plasma-membrane marker enzyme 5'-nucleotidase, expressed per mg of plasma-membrane protein, was similar, indicating that the degree of purification of the IBAT plasma membrane was independent of the experimental conditions (results not shown).

Table 1 shows the results obtained from the saturation curves of [^{125}I]cyanopindolol binding and from the stimulation of adenylate cyclase. The maximal number of β -adrenergic binding sites (B_{max}) per IBAT was not modified in obese rats as compared with that in lean controls. It was not changed by Ro 16-8714 treatment in lean rats, but it was increased 2.8-fold in obese rats. The (–)-isoprenaline- and NaF-stimulated adenylate cyclase activities per IBAT were decreased by 40 and 38% respectively in obese rats. They were not changed by Ro 16-8714 treatment in lean rats, but were increased 2.5- and 2.0-fold in the presence of saturating concentrations of (–)-isoprenaline and of 10 mM-NaF respectively in obese rats. Isoprenaline-stimulated adenylate cyclase activity, expressed as a fraction of the NaF-stimulated adenylate cyclase activity, was 77 ± 2 and $71 \pm 4\%$ ($n = 3$) for lean and obese rats respectively. Ro 16-8714 did not modify this value significantly in lean rats, but increased it to $90 \pm 7\%$ ($n = 3$, $P < 0.05$) in obese rats.

The K_D for [^{125}I]cyanopindolol binding and the K_{act} for (–)-isoprenaline stimulation of adenylate cyclase activity were unchanged in obese compared with normal rat IBAT plasma membranes (K_D 34 ± 5 pmol/l, $n = 5$; K_{act} 1.4 ± 0.2 $\mu\text{mol/l}$, $n = 7$) and were not modified by Ro 16-8714 treatment (results not shown).

The increase in the amount of plasma membrane per IBAT induced by Ro 16-8714 in obese rats cannot account for the total increase in [^{125}I]cyanopindolol binding and (–)-isoprenaline-stimulated adenylate cyclase activity. As shown in Table 2, in fact the activities, expressed per mg of IBAT plasma-membrane protein, of [^{125}I]cyanopindolol binding and of (–)-isoprenaline-stimulated adenylate cyclase activity were also increased by Ro 16-8714 treatment in obese rats, by 1.6- and 1.5-fold respectively.

The effect of Ro 16-8714 on the steady-state level of UCP mRNA was also measured in lean and obese rats. Northern blots were hybridized with the UCP cDNA probe and with the total mitochondrial DNA probe. The mRNAs were quantified by densitometric scanning of non-saturated autoradiograms and the results expressed

Table 1. Effect of Ro 16-8714 administration to lean and obese (*fa/fa*) rats on [^{125}I]cyanopindolol binding, (–)-isoprenaline-stimulated and NaF-stimulated adenylate cyclase, expressed per IBAT

The results are means $\pm$ S.E.M. for the numbers of experiments in parentheses. Isolation of IBAT plasma membranes and assays are described in the Materials and methods section. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus corresponding lean; † $P < 0.05$, †† $P < 0.01$, Ro 16-8714-treated versus corresponding control.

	Binding (B_{max}) (fmol/30 min per IBAT)	Adenylate cyclase activity (V_{max}) (pmol of cyclic AMP/min per IBAT)	
		+ isoprenaline	+ NaF
Lean	61.4 ± 8.4 (3)	144 ± 9 (3)	194 ± 6 (3)
+ Ro 16-8714	57.2 ± 8.5 (3)	108 ± 21 (3)	149 ± 16 (3)
<i>fa/fa</i>	79.3 ± 11.1 (4)	86.5 ± 18.0 (4)*	121 ± 18 (4)**
+ Ro 16-8714	220 ± 27 (6)***††	217 ± 7 (3)*††	241 ± 28 (3)*††

Table 2. Effect of Ro 16-8714 administration to obese (*fa/fa*) rats on [125 I]cyanopindolol binding, (–)-isoprenaline-stimulated and NaF-stimulated adenylate cyclase, expressed per mg of IBAT plasma-membrane protein

The results are means $\pm$ S.E.M. for the numbers of experiments in parentheses. The values shown are those of Table 1, but expressed per mg of IBAT plasma-membrane protein. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, Ro 16-8714-treated versus corresponding control.

	Binding ($B_{\max}$) (fmol/30 min per mg)	Adenylate cyclase activity ($V_{\max}$) (pmol of cyclic AMP/min per mg)	
		+ iso- prenaline	+ NaF
<i>fa/fa</i>	233 $\pm$ 33 (4)	231 $\pm$ 33 (4)	326 $\pm$ 28 (4)
+ Ro 16-8714	381 $\pm$ 37 (6) $\dagger\dagger$	380 $\pm$ 36 (3) $\dagger$	424 $\pm$ 46 (3)

as the ratio of UCP mRNA versus the sum of the major mitochondrial mRNA signals. It was found that Ro 16-8714 administration increases UCP mRNA specifically by factors of 3.1 ± 0.2 ($n = 5$) and 2.7 ± 0.3 ($n = 5$) in lean and obese rats respectively. The difference between the two effects is not significant.

DISCUSSION

Neither the affinity nor the capacity of [125 I]cyanopindolol binding to IBAT plasma membranes was modified in obese rats as compared with lean controls. These results agree with those of Raasmaja & York [7], who showed that the number of β -receptors per unit of BAT membrane was identical in young 6-week-old lean and obese (*fa/fa*) Zucker rats. It is noteworthy that in adult (4–6-month-old) obese (*fa/fa*) rats the number of β -receptors per brown adipocyte was much decreased as compared with that of lean rats [8,9]. The activity of (–)-isoprenaline- or NaF-stimulated adenylate cyclase in IBAT plasma membranes was decreased by obesity. Results already reported for *ob/ob* mice are controversial, showing either no difference [23] or a decrease [24] in β -adrenergic-agonist-stimulated adenylate cyclase values as compared with lean animals.

The acute treatment with Ro 16-8714 was found to decrease IBAT wet weight in lean and obese rats. This effect is probably due to a decrease in IBAT lipid content analogous to that described by Wilson *et al.* [25] during an acute treatment with another thermogenic β -agonist, BRL 26830A.

Ro 16-8714 treatment was found to increase the amount of plasma membrane per IBAT in obese (*fa/fa*) rats [13]. An acute sympathetic stimulation of IBAT by 4 days of cold exposure has been reported to increase the growth of the tissue in normal rats, as evidenced by a markedly enhanced mitotic activity in brown-adipocyte precursor cells [26]. It is noteworthy that Ro 16-8714, which could be expected to produce a stimulation of IBAT analogous to that of cold exposure, induces trophic effects on this tissue, characterized by an increase in plasma membrane and cytochrome oxidase content only in the obese rats [13].

Ro 16-8714 treatment markedly increased the total number of β -receptors and slightly increased the receptor density, these effects being observed only in obese rats. Studies of the effects of exposure to cold on β -receptors in BAT gave conflicting results. In normal rats, after periods of exposure to cold ranging between 2 days and 5 weeks, β -receptor density, whether expressed per mg of membrane protein or per cell, was reported to be decreased [27–30], unchanged [31] or even increased [32] as compared with that of rats kept at room temperature. Cold-acclimation was found to increase the total number of β -receptors per IBAT, owing to tissue hyperplasia [29]. In a recent study, comparing lean and obese (*fa/fa*) Zucker rats Raasmaja & York [7] reported that cold-acclimation for 2 weeks causes a slight increase in β -receptor density in both genotypes. Ro 16-8714 induces similar effects within 30 h, but only in obese rats.

Ro 16-8714 treatment markedly increased the (–)-isoprenaline-stimulated adenylate cyclase activities per mg of plasma membrane or per IBAT, these effects being observed only in obese rats. The increase in specific activity might be partly due to the observed increase in β -receptor density and/or to an improved coupling between the β -receptor and the catalytic unit. The results show, indeed, that Ro 16-8714 treatment does not increase significantly the specific activity of NaF-stimulated adenylate cyclase, whereas it increases the (–)-isoprenaline-/NaF-stimulated adenylate cyclase activity ratio. Studies of the effects of exposure to cold on the adenylate cyclase activity in BAT also gave conflicting results. In normal rats, cold exposure and cold-acclimation were reported to cause either a desensitization of BAT, characterized by a decrease in the catecholamine/forskolin-stimulated adenylate cyclase activity ratio or by a decrease in the adenylate cyclase activation by catecholamines [28,33], or, on the contrary, a supersensitivity characterized by an increase in adenylate cyclase activation by catecholamines [31,32]. In the present study, Ro 16-8714 was found to induce effects comparable with some of those induced by exposure to cold reported in the literature [31,32], but only in obese rats.

The data show that the classical model of desensitization described *in vitro* in amphibian erythrocytes or in various cultured cell lines [34] cannot be extrapolated as such *in vivo*.

Why Ro 16-8714 only acts on obese, but not on lean, animals, and why it does so in a way which, concerning the β -receptor–adenylate cyclase couple, is more suggestive of the induction of a supersensitivity than of a desensitization, is not clear, and deserves further study. It might be related to the existence of an atypical β -receptor in BAT [35], and to possible modification of some of its characteristics in obese animals.

Cold exposure and BRL 26830A were found to result in a rapid increase in the expression of UCP mRNA in lean and in obese (*fa/fa*) rats and *ob/ob* mice [36]. The increase owing to cold exposure was reported to be attenuated in obese (*ob/ob*) mice as compared with their lean littermates [37]. In the present study, the stimulation by Ro 16-8714 of UCP mRNA expression, unlike that of the plasma-membrane β -adrenergic-effector system, was found to occur in both lean- and obese- (*fa/fa*) rat IBAT. The higher level of (–)-isoprenaline-induced cyclic AMP production in obese-rat IBAT under Ro 16-8714 treatment is not correlated with a higher UCP mRNA

expression *in vivo*. This might be due to the fact that a complete stimulation of UCP mRNA transcription is obtained during the first few hours of Ro 16-8714 treatment, i.e. before the increase in the plasma-membrane β -adrenergic system takes place.

The present study describes a paradoxical action of a novel β -adrenergic agonist in the β -receptor-adenylate cyclase couple of obese (*fa/fa*)-rat IBAT. It suggests that desensitization is not an obligatory consequence of β -adrenergic stimulation *in vivo*. The latter, in some instances, can elicit a supersensitivity.

We greatly appreciated the excellent technical help of Miss C. Colomb. We are very grateful to Dr. H. Blanc, University of Paris-Sud, Orsay, who provided the pST 41 probe. This work was supported by grants N° 3.638.087 and 3.822.086 from the Swiss National Science Foundation, and by grants-in-aid from Sandoz Stiftung zur Förderung der Medizinisch-Biologischen Wissenschaften (Basel), from the Carlos et Elsie de Reuter Foundation and from the Société Suisse d'Assurances générales sur la vie humaine pour la santé publique et les recherches médicales.

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Received 24 November 1988/10 March 1989; accepted 28 March 1989